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14. ABSTRACT: Purpose: To explore the effects of exercise training on tumor vascularity and response to neoadjuvant therapy in operable breast cancer in a phase I-II randomized design. Method: Using a two-armed, prospective, randomized design, potential participants will be identified and screened for eligibility via medical record review of patients scheduled for their primary neoadjuvant chemotherapy treatment consultation at DUMC. Following the successful completion of all baseline assessments participants will be randomly assigned to an exercise or control group. Participants assigned to combined exercise training and chemotherapy will perform an individualized exercise training program consisting of three cycle ergometry sessions per week at approximately 60-80% of VO2peak on nonconsecutive days for the duration of neoadjuvant chemotherapy (approximately 12 weeks). Most Significant Research Finding During Reporting Period: As per our statement of work, human ethical approval was obtained (Task 1); all exercise testing / training procedures and clinical protocols were clarified (Task 2), and data collection has been initiated (Task 3). To date, all 3 patients in phase I of the study have been enrolled and no dose-limiting toxicities have been identified with acceptable exercise adherence (>60%), thus phase II has been initiated.					
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Introduction:

Expression of some receptor tyrosine kinases has been shown to correlate with outcome of breast cancer patients, making these receptors useful markers for prognosis and promising targets for therapeutics. The Met receptor has been shown to have predictive value in breast, gastric, cervical and head and neck carcinomas. A previous study showed antibodies to the cytoplasmic domain of Met to be associated with worse outcome in node-negative breast carcinomas, whereas, antibodies to the extracellular domain do not predict patient outcome. Upon further inspection, it was observed that antibodies to the intracellular domain of Met showed localized expression in the nucleus. The purpose of this research was to confirm the presence of Met in the nucleus, determine the origin of nuclear Met (alternative splice, full-length receptor or cleavage product) and identify its role in the pathway activated by the ligand for Met, the Hepatocyte Growth Factor.

Body:

The goal of this study was to verify nuclear expression of the Met receptor, the mechanism by which Met translocates to the nucleus and its relationship to HGF-Met signaling.

The original statement of work was as follows:

Task 1. To confirm nuclear localization of the cytoplasmic domain of the Met receptor in HMEC cells, Months 0-24:

- a. Construction and transfection of GFP-tagged Met cytoplasmic domain, Months 0-3
- b. Subcellular fractionation, Months 3-6
- c. Identification of Met antibodies for use on tissue microarray of normal and cancerous cells, Months 12-24

Task 2. Definition of the domain responsible for translocation to the nucleus, Months 6-30):

- a. Construct and transfection of truncated versions of GFP-tagged cytoplasmic Met, Months 6-12
- b. Subcellular fractionation, Months 12-18
- c. Characterization of turnover rate, 18-30

Task 3. Definition of the cleavage domain and cleavage site, Months 18-36:

- a. Tag full length Met and transfect into cells to verify cleavage, Months 18-21
- b. Bioinformatics to determine and test protease candidates, Months 21-27
- c. Create mutant Met unable to be cleaved, Months 27-30
- d. Characterize mutant and wild type effect on cells using growth assays, Months 30-36+

The aim of Task 1 was to confirm nuclear localization of Met. Immunohistochemistry, using an anti-peptide polyclonal antibody to the C-terminus of the Met receptor, shows Met in the cytoplasm and nucleus of normal and cancerous tissues rather than the previously reported

predominantly membrane staining (Figure 1a)(1,2). Normal colon, skin and testis show similar nuclear staining of Met in germinal tissue layers and aberrant expression throughout cancerous tissue. The transition to Met over-expressing cancers is most dramatically seen in lymphoma tissue where Met is completely absent in the normal lymph node tissue and localized almost exclusively to the nucleus in cancerous tissue.

To confirm that the nuclear localization of Met expression was not an artifact seen only in formalin fixed, paraffin embedded tissue, a series of cell lines were stained with four different antibodies to the cytoplasmic domain. The antibodies selected were polyclonal antibodies C12, C28, and CVD13 to peptides very near or including the C-terminus of Met, and 3D4, a monoclonal antibody to the tyrosine kinase domain. Mammary epithelial lines HMEC and MCF10-A, melanoma cell lines Mel1241 and Mel1335 (data not shown), and epidermoid carcinoma line A431 (Figure 1b) all showed nuclear expression of Met to varying degrees depending on the antibody used. HEK293 and NIH3T3 cell lines express low levels of Met yet also showed nuclear staining equivalent to those known to express higher levels of Met. DO24, a monoclonal antibody to the extracellular domain of Met, shows membranous staining of HMEC, MCF-10A, HEK293 and A431 cell lines only in dense regions of the cell colony.

Since nuclear staining of Met was seen in cell lines previously considered low expressers or negative for Met, a northern blot was done to confirm the presence of Met in these cell lines. Using a radioactively-labeled probe representing 450 base pairs at the 3' end of the translated sequence of Met, a 9.5kb transcript was detected in all cell lines. The smaller 7.5kb variant of Met was also seen in all cell lines except NIH3T3 and Mel1335 (Figure 2). No smaller isoforms were seen. Thus, although some lines (NIH 3T3) have previously been used as negative controls for Met transfection (3), full length message is observed for each cell line. Furthermore, this also suggests that the protein is not derived from a novel alternative splicing variant.

To determine if Met is present in the nucleus, cell fractionation was done to separate the nuclear fraction from the membranous and cytoplasmic fractions using standard sucrose gradient-based methods. The epidermoid carcinoma cell line A431 was used as a model. Separation of the cytoplasm from the nucleus resulted in a 60kDa protein that is reactive with Met antibody localizing to the nuclear fraction, while the 145kDa Met receptor remained in the cytoplasmic fraction in both A431 (high Met expresser) and HEK293 (low Met expresser) cell lines (Figure 3a). Tubulin is shown as a control for the cytoplasmic fraction and lamin serves as a control for the nuclear fraction. Since the 60 kD fragment has not previously been reported, we used a series of two polyclonal antibodies and one monoclonal antibody to prove the band is Met and not a cross reacting protein. Western blotting of A431 lysates from this line showed the expected band at 145 kDa and no band at 60kDa. Immunofluorescence detected low levels of nuclear Met in A431 cells suggesting that the nuclear fragment may need to be enriched in some manner to detect it on a western blot, this is in contrary to subcellular fractionation results as fractionation served to enrich the amount of nuclear protein seen on a western blot. When A431 cells were serum starved and treated with ALLN, a proteasome inhibitor, a band that co-migrates with 60kDa markers is detected by three different antibodies to Met (Figure 3b). To further prove specificity we immunoprecipitated Met from total cell lysates of ALLN treated and untreated A431 cells as well as untreated HEK293 cells and NIH3T3 cells. All cell lines show the cleaved Met precursor (190kDa), full length Met (145kDa) and a band of 60kDa (Figure 3c)

when immunoprecipitated with either a monoclonal or a polyclonal Met antibody. As seen in subcellular fractionation, the presence of the 60kDa fragment in untreated cells is again due to the enrichment capability of the assay. Immunoprecipitation of NIH3T3 cells, which do not express Met at the membrane, does not pull down full length Met when immunoprecipitated with the C12 antibody, however the 3D4 antibody pulls down the uncleaved Met precursor. Additionally, the 3D4 antibody shows a band slightly higher than the 60kDa fragment that also appeared faintly in the no antibody control lane. Together, this data supports the presence of a 60kDa fragment of Met in the nucleus.

Task 2 was to define the domain responsible for nuclear translocation. To verify that a region of the cytoplasmic domain is present in the nucleus, Met and N-terminally truncated forms of the cytoplasmic domain of Met were cloned into a GFP fusion vector so that GFP was located on the C-terminus of the protein. A full length Met protein fusion, a fusion with the cytoplasmic domain truncated at the transmembrane/intracellular junction (K956), three constructs truncated after tyrosine residues in the juxtamembrane region (D972, R1004, P1027), the tyrosine kinase domain (I1084) and a truncated tyrosine kinase domain (L1157) were constructed. Each Met construct had a predicted molecular weight of 49, 47, 43, 41, 35 and 27 kDa, respectively, the GFP tag is an additional 30kDa. All of the constructs shown in a schematic in Figure 4a, were then transiently transfected into HEK293 cells. A Western blot of HEK293 cells transiently transfected with the cytoplasmic domain of Met confirmed the presence of the GFP-tagged Met construct (Figure 4b). The full length Met-GFP fusion localized to the plasma membrane of HEK293 cells as expected (Figure 4c). Surprisingly, this construct has not been seen in the nucleus under any conditions, including treatment with HGF. The absence of the Met-GFP construct from the nucleus may be due to the construction of an unnatural state of forced overexpression as nuclear expression of Met is not readily seen in HT-29 cells which overexpress Met (data not shown). As we have yet to determine the mechanism by which Met enters the nucleus another possibility for the lack of nuclear Met-GFP is that the transfected cells were not properly stimulated to express nuclear Met-GFP. However, constructs of the cytoplasmic domain of Met localized to the nucleus of cells. Loss of the juxtamembrane region caused Met-GFP tyrosine kinase domain constructs to be partially excluded from the nucleus of cells and loss of the N-terminus of the tyrosine kinase domain led to complete exclusion from the nucleus. (Figure 4c). The same localization results were observed in transfections of MCF-10A cells (data not shown). Transient transfections of Met-GFP constructs confirm the ability of the cytoplasmic domain of Met to enter the nucleus. The region of the juxtamembrane domain preceding the tyrosine kinase domain (P1027-I1084) appears to be necessary for nuclear localization of the cytoplasmic domain. As we have yet to identify the conditions required for stimulating nuclear translocation of the Met-GFP construct, we have not been able to investigate turnover rate of the fragment.

The goal of Task three was to further describe the cleavage site of Met and characterize its function in the HGF-Met signaling pathway. The first aim of task 3 was to create a full-length Met-GFP construct. The results of this experiment were described under task 2 in relation to the truncated Met-GFP constructs. As for the full length Met-GFP construct, it localized exclusively to the membrane and was not seen in the nucleus even after treatment with HGF. HGF is the ligand for Met, and its interaction with the extracellular domain of Met triggers dimerization and phosphorylation of the receptor and activation of a number of downstream signaling pathways

including the MAPK pathway (4). In order to confirm the effect of HGF on the endogenous 60kDa fragment we treated two model cell lines with HGF over a 24 hour time course. Treatment with HGF did not induce the appearance of the 60kDa fragment. Figure 5 shows that although HGF is activating downstream signaling, as shown by phosphorylation of ERK1/2, the 60kDa fragment is not induced. However, treatment with ALLN, which inhibits proteolysis, substantially increases the amount of the 60kDa fragment in both cell lines in both the presence and absence of HGF (Figure 5). As we have not been able to stimulate cleavage of the full length construct we attempted to identify the cleavage site by immunoprecipitating the nuclear fragment from HEK293 cells. This has been unsuccessful in that we have been unable to immunoprecipitate sufficient quantities of the cleaved fragment for sequencing. Attempts at making an uncleavable Met have not been made at this time as we have not yet identified the cleavage site.

Although there has been no progress on identifying a stimulant for cleavage of the Met-GFP construct, an endogenous model for Met going from the nucleus to the membrane and back to the nucleus was observed and has been at the forefront of our recent studies. It has been observed that nuclear expression of Met is cell density dependent. CaCo2 cells undergo cell maturation and differentiation as a function of time and density when grown in culture (5,6). Preconfluent CaCo2 cells show nuclear and cytoplasmic expression of Met as seen in Figure 6a. CaCo2 cells matured for three days past confluence show a more distinct membranous as well as cytoplasmic expression of Met (Figure 6b). Similar relocalization patterns were obtained with A431 (data not shown), however, it required A431 cells to be grown for a minimum of ten days past confluence to detect regions of membranous Met expression. More recently, we have observed this phenomenon in a number of breast cell lines, including MCF-7 cells, if the cells are allowed to grow densely. This suggests that nuclear expression of Met may be detected only in cells in a state of rapid proliferation or in a less differentiated state, a hypothesis consistent with the expression patterns of Met in proliferative areas of normal tissues in Figure 1a and in moderately or poorly differentiated cancers. In accordance with this hypothesis, a wound healing assay on these highly confluent cells causes Met to translocate from the membrane to the nucleus at the wound edge in both CaCo-2 and MCF-7 cells (Figure 7). Bioinformatics suggest a possible gamma-secretase cleavage site located in the transmembrane region of Met and attempts at inhibiting this phenomenon with gamma-secretase and caspase inhibitors are underway using wound healing as a model. Meanwhile, in an effort to further understand the effect of nuclear Met on both wound healing and the HGF pathway, CaCo-2 and MCF-7 cells were treated with the Pfizer small molecule Met inhibitor PHA-665752 which inhibits phosphorylation by acting as an ATP competitor and/or HGF and wounded by creating a circular punch. Activation by HGF and inhibition by PHA-665752 were confirmed by western blot in Figure 8a. Figure 8b shows that HGF increases wound healing in comparison to the untreated cells and the Met inhibitor, inhibits HGF-induced wound healing. Cells expressing nuclear Met at the wound edge after 24 hours of treatment were counted and normalized to the area of the wound at time 0. Both CaCo-2 and MCF-7 cells show a decrease in nuclear expression of Met at the wound edge after treatment with the Met inhibitor as seen in Figure 8c. HGF treatment had no effect on the number of nuclear Met expressing cells confirming the western blot data in Figure 5 which suggested that nuclear expression of Met was independent of HGF activation. Interestingly, this data suggests that ligand-independent Met activation is necessary for nuclear localization of Met.

Key Research Accomplishments:

- A 60kDa fragment of the Met receptor localizes to the nucleus of normal and cancerous cells, as shown by C-terminal antibody staining, cell fractionation and transfection of Met-GFP constructs.
- The juxtamembrane region from P1027 to I1084 is required for nuclear localization of Met-GFP constructs.
- Perturbation of mature epithelial sheets by a wound healing assay is associated with nuclear translocation of the Met fragment.
- Translocation of Met to the nucleus requires ligand-independent activation as highlighted by wound healing experiments with the small molecule inhibitor PHA-665752.

Reportable Outcomes:**Publications:**

Pozner-Moulis, S., D.J. Pappas, and D.L. Rimm, *Met, the hepatocyte growth factor receptor, localizes to the nucleus in cells at low density*. Cancer Res, 2006. **66**(16): p. 7976-82.

Abstracts:

Sharon Pozner-Moulis, Derek Pappas, David L. Rimm, *Nuclear localization of the Met receptor*, American Association for Cancer Research, 2006, Washington D.C.

Sharon Pozner-Moulis, Derek Pappas, David L. Rimm, *Nuclear localization of the Met receptor*, Federation of American Societies for Experimental Biology, 2005, Tucson, Arizona

Sharon Pozner-Moulis, Derek Pappas, David L. Rimm, *Met Nuclear Localization and Signaling in Breast Cancer*, Era of Hope Department of Defense Breast Cancer Research Program Meeting, 2005, Philadelphia, Pennsylvania

Sharon Pozner-Moulis, Derek Pappas, David L. Rimm, *Met Nuclear Localization and Signaling in Breast Cancer*, San Antonio Breast Cancer Symposium, 2004, San Antonio, Texas

Personnel Receiving Pay:

Sharon Pozner-Moulis

Conclusion:

Here, we demonstrate for the first time that a C-terminal fragment of the HGF receptor Met localizes to the nucleus in a ligand-independent manner. Using a series of monoclonal and polyclonal antibodies to the C-terminus of Met, we show that Met expression is present not only at the membrane, but also in the cytoplasm and nucleus in seven cell lines and predominantly in germinal regions in a range of tissues from normal organs. Nuclear translocation is reconstituted by transfection of GFP fusions with Met that migrate or do not migrate to the nucleus, dependent on the composition of the fusion. Cell lines expressing nuclear Met have either a mesenchymal phenotype or are on the leading edge of epithelial clusters or coincide with a less differentiated state. The maturation of the Caco2 cells provides the best model to illustrate this hypothesis.

All cell lines expressing Met in the nucleus show Met transcripts by Northern Blot. Western blotting reveals a 60kDa band recognized by antibodies to the C-terminus of Met that localizes to the nucleus. The appearance of a smaller protein recognized by Met antibodies in the nucleus and the lack of a smaller transcript by Northern Blot suggests that this fragment is derived from the full length Met receptor by a processing event. The increase in amounts of the fragment in the presence of ALLN provides further evidence for a processing event. Although no nuclear localization sequence has been identified, serial deletion constructs of Met isolate a region of the juxtamembrane domain (P1027-I1084) that is required for nuclear localization of the cytoplasmic fragment of Met. This fragment is not an alternative splice as shown by Northern blotting and is most likely the product of a cleavage event as the full length Met-GFP fusion does not localize to the nucleus.

This work has identified a possible “noncanonical” pathway for the Met receptor. Met is well known for its HGF-induced functions in cell proliferation, motility, migration and invasion; functions key for both embryonic development and cancer progression. It has previously been shown that the Met receptor can induce wound healing independently of HGF treatment (7) however the details of how Met may induce wound healing independent of HGF is unknown. Here, for the first time we show that a fragment of Met localizes to the nucleus at a steady state in immature cells. Met controls aspects of cell differentiation (8); we show that nuclear translocation of Met is inhibited by early stages of cell maturation in a Caco2 cell line model for cell differentiation yet nuclear translocation of Met can be induced by injury to the maturing cell monolayer. Together, these results suggest that nuclear localization of Met occurs in cells undergoing continuous growth and motility as seen by our studies with Caco-2 cells as well as immunohistochemical staining where nuclear expression of Met is seen in proliferative regions of normal and cancerous tissue.

Many pharmaceutical companies have Met inhibitors in Phase I clinical trials, yet the story of Met activity is just beginning to unfold. Using the Pfizer small molecule inhibitor to Met, it is shown that the inhibitor decreases the amount of nuclear Met expressing cells. This suggests a role for an activated Met in wound healing independent of HGF activation. As targeted therapies are designed it becomes critical to understand the pathways which these drugs are targeting, here we show a novel ligand-independent pathway for Met nuclear localization that is not understood and may inadvertently be targeted by novel cancer therapeutics. To further address this lack of knowledge in the function of nuclear Met, our lab is undertaking a chromatin immunoprecipitation project where the DNA that is directly or indirectly pulled down by the Met

antibody will be sequenced by 454 Sciences (Branford, CT) in order to identify possible transcriptional targets of Met in the nucleus. This experiment will hopefully lead to a better understanding of the Met pathway as well as knowledge of what these targeted therapeutics are targeting downstream of Met. Other future directions include determining the mechanism by which Met goes to the nucleus using wound healing as a model, from cleavage to entry into the nucleus and possibly direct or indirect DNA binding as this could lead to the discovery of other potential drug targets as well as give a more accurate picture of the ligand-independent functions of the Met receptor.

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Appendix 1: Figure Legends

Figure 1. **Met localizes to the nucleus in normal and cancerous tissues and cell lines.**

a) Immunohistochemistry of normal and cancerous tissue samples on a multi-tumor tissue microarray using the C28 antibody to the C-terminus of Met.

b) Immunofluorescence of cell lines using 4 different antibodies to the cytoplasmic domain of Met show varying levels of membranous, cytoplasmic, and nuclear expression.

Immunofluorescence using an antibody to the extracellular domain of Met (DO24) shows cytoplasmic and membranous expression. The names of the antibodies used are inset in the leftmost panel for each row. Images were taken at 40x (MCF-10A and HMEC images were taken at 20x).

Figure 2. **Validation of Met RNA expression in cell lines.** Northern blot probed with a region of the tyrosine kinase domain of Met reveals known 7.5 and 9.5 kb Met transcript in all cell lines tested, including low expressing cell lines such as NIH3T3. No novel alternative splices of Met were detected.

Figure 3. **A 60 kDa fragment of Met localizes to the nucleus.** (a) Cellular fractionation of HEK293 (lanes 1 and 2) and A431 cells (lanes 3 and 4). Cytoplasmic fraction (C), Nuclear fraction (N). The Met receptor (145kD) localizes to the cytoplasmic fraction and a novel 60kD fragment recognized by the Met antibody localizes to the nuclear fraction. Purity of fractions were determined by the presence of tubulin (cytoplasmic marker) and lamin (nuclear marker) (b) A431 total cell lysates treated with vehicle or 250 uM ALLN show the 60kD fragment of Met after treatment with ALLN. Western blots were probed with 3 different antibodies to the C-terminus of Met as labeled, C12, CVD13 and 3D4. (c) Immunoprecipitations of A431 cells, A431 cells treated with 250 uM ALLN, HEK293 cells and NIH3T3 cells (low expresser) all show a 60kD fragment recognized by antibodies to the C-terminus of Met. Antibodies used to precipitate and Western blot for each panel are indicated by IP and WB respectively.

Figure 4. **Met-GFP fusion proteins identify a region of Met required for nuclear translocation.** (a) N-terminal deletions of the cytoplasmic domain of Met were constructed and inserted into the pEGFP plasmid such that GFP is expressed on the C-terminus of the construct. Truncations were made following the transmembrane domain (Cyto), each of three tyrosine residues in the juxtamembrane domain (Jxtm1, Jxtm2, Jxtm3), at the interface of the juxtamembrane and tyrosine kinase domains (Tkd1) and after the first structural lobe/activation loop of the tyrosine kinase domain (Tkd2). (b) Western blot of HEK293 cells transiently transfected with CytoMet-GFP shows successful transfection of the construct recognized by both GFP and the C12 Met antibody. (c) Confocal images of Met-GFP constructs transfected into HEK293 cells. The full-length Met-GFP fusion localizes to the membrane as expected. Constructs to the cytoplasmic domain and the three juxtamembrane deletions accumulate in the nucleus of these cells. The tyrosine kinase domain (Tkd1) appears non-specific in its localization and the truncated tyrosine kinase domain (Tkd2) is completely excluded from the nucleus. Bar, 5µm.

Figure 5. **The nuclear fragment of Met is independent of HGF activation.** A431 and CaCo2 cells were treated with 250uM ALLN, 20 ng/ml HGF or pretreated with ALLN for 30 minutes

followed by ALLN/HGF treatment for 1, 6, or 24 hours. HGF treatment had no effect on the presence of the 60kD fragment when compared to the control in lane 1. Activation of Met by HGF was verified by phosphorylation of ERK. ALLN has function as both a protease and phosphatase inhibitor which may explain why pERK is present in all ALLN treated lysates. Western blots using lysate from the same experiment were pieced together as indicated.

Figure 6. Met localization in CaCo2 cells depends on cell maturation. CaCo2 cells at low (a) and high density (b) were stained for nuclei (DAPI), the cell membrane (E-cadherin) and Met (CVD13). Met is expressed in the nucleus and cytoplasm at low density (a) and in the membrane and cytoplasm at high density (b) as seen in 20x images (upper panel), 60x images (middle and lower panels) and deconvolution images (lower panels) created using the DeltaVision system. Bar, 8.6µm.

Figure 7. Nuclear expression of Met in wound healing. CaCo-2 (left panel) and MCF-7 (right panel) cells were grown for 5 days after reaching confluence and wounded with a p200 pipet tip. Met staining with the CVD13 antibody reveals nuclear expression of Met at the wound edge and membranous expression surrounding the wound at 10X.

Figure 8. Effect of HGF and Met inhibitor PHA-665752 on wound healing. a) Immunoprecipitation of CaCo-2 cells after treatment with HGF (20ng/ml) for 60 minutes or PHA-665752 (1µM) for 60 minutes or 60 minutes prior to HGF treatment. Lane 1 is a beads only control. HGF increased phosphorylation of Met (upper panel) and was inhibited in the presence of PHA-665752. b) CaCo-2 and MCF-7 cells were grown for 5 days after reaching confluence and wounded with a p200 pipet tip. Cells were treated with PHA-665752 (1µM) for 1 hour prior to wounding and HGF (50ng/ml) at time of wounding. Area of the wound was measured at time 0 (wounding) and 24 hours after wounding. c) The number of cells expressing nuclear Met at the wound edge was counted at 24 hours after treatment and normalized to the size of the wound at time 0. 10 fields of each treatment were measured; error bars represent 1 standard deviation.

Figure 1a

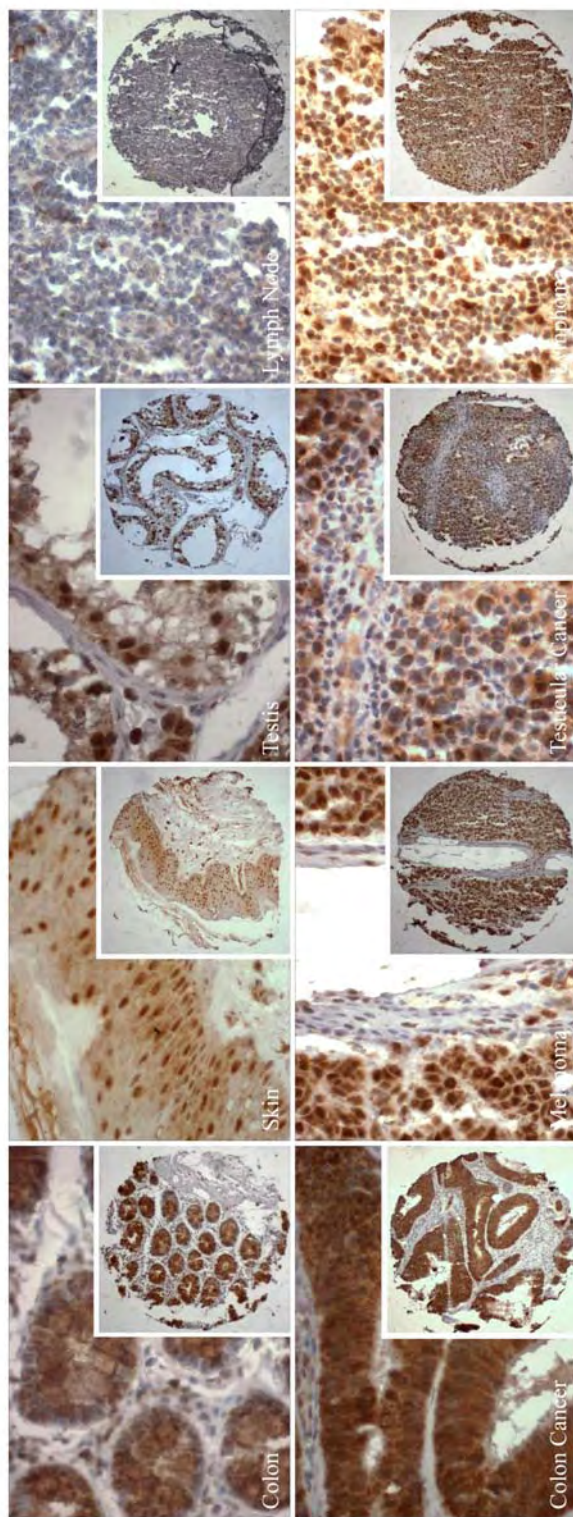


Figure 1b

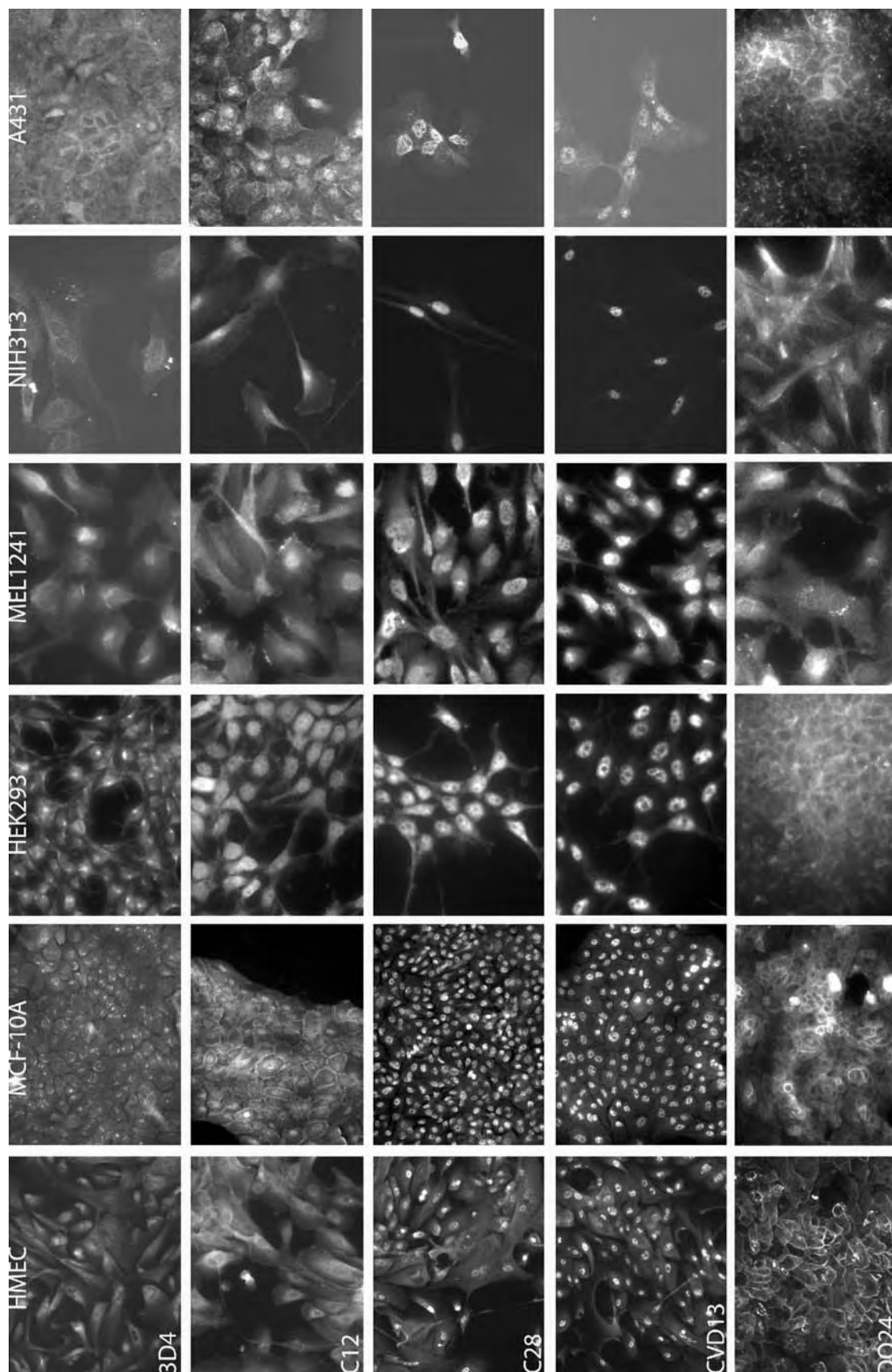


Figure 2

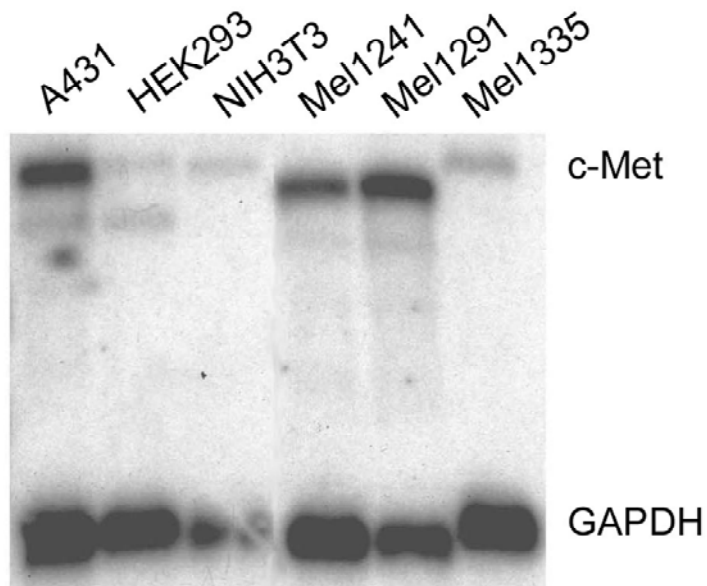


Figure 3

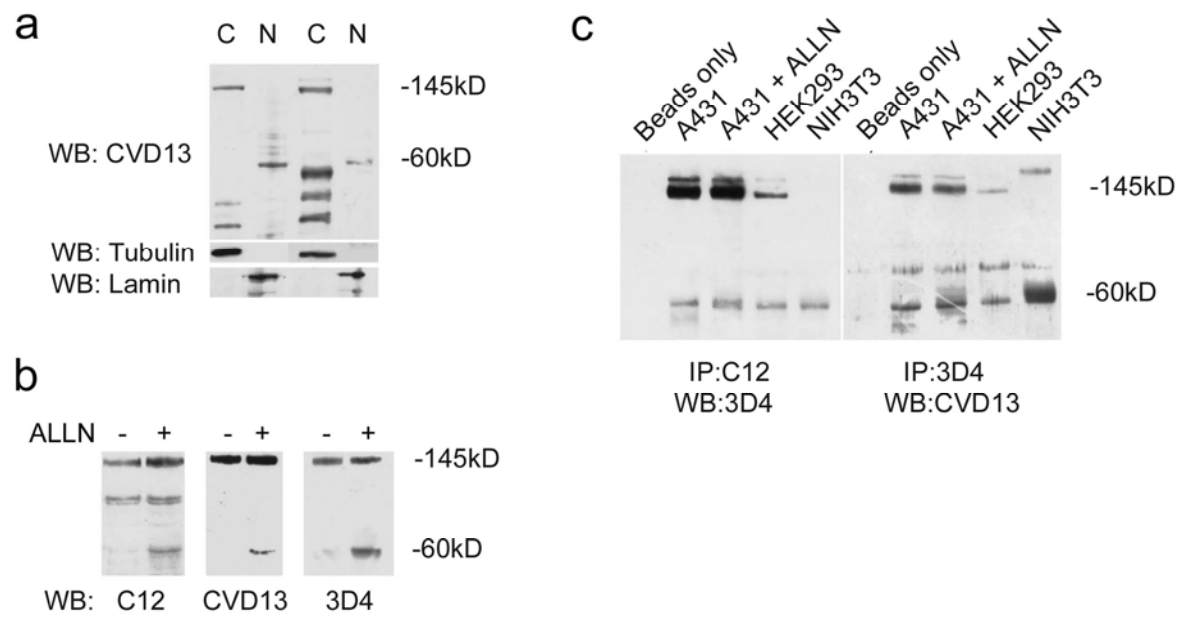


Figure 4

a



b

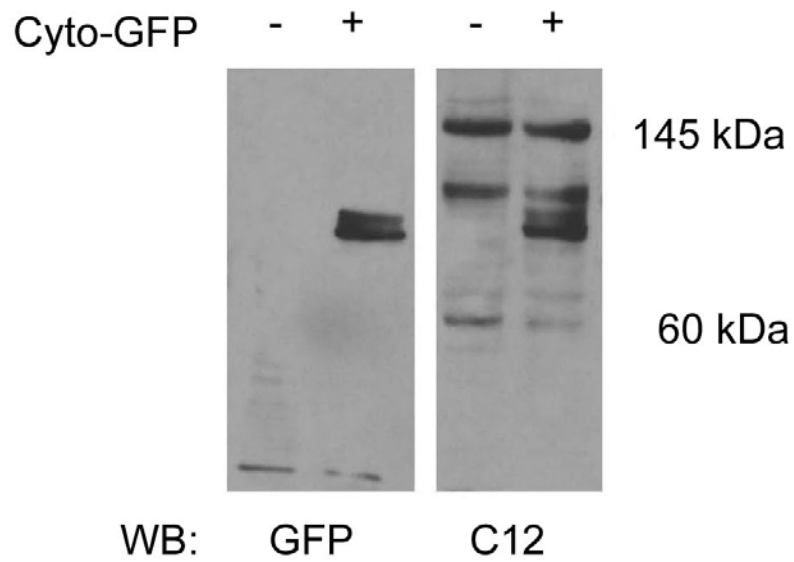


Figure 4c

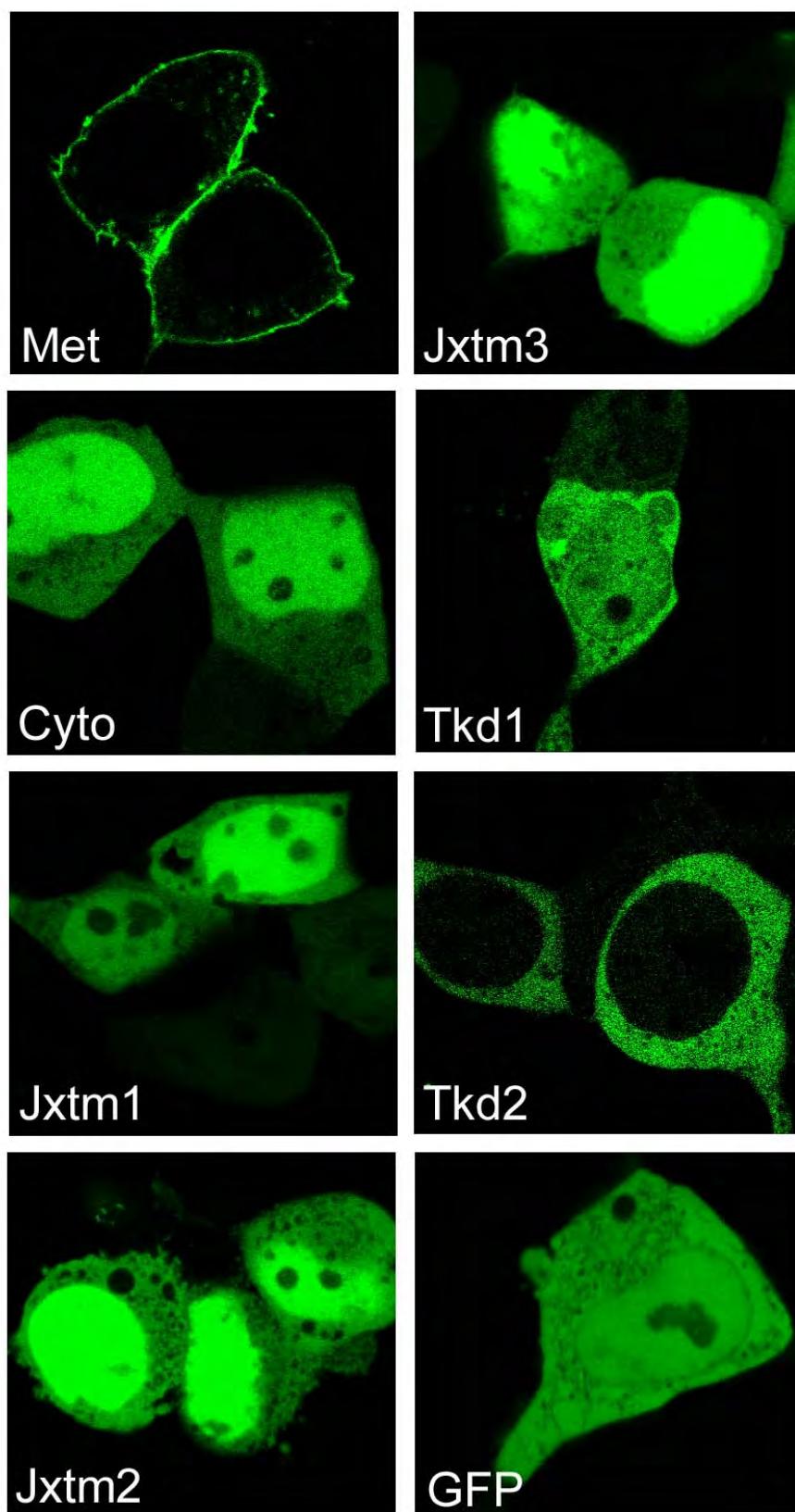


Figure 5

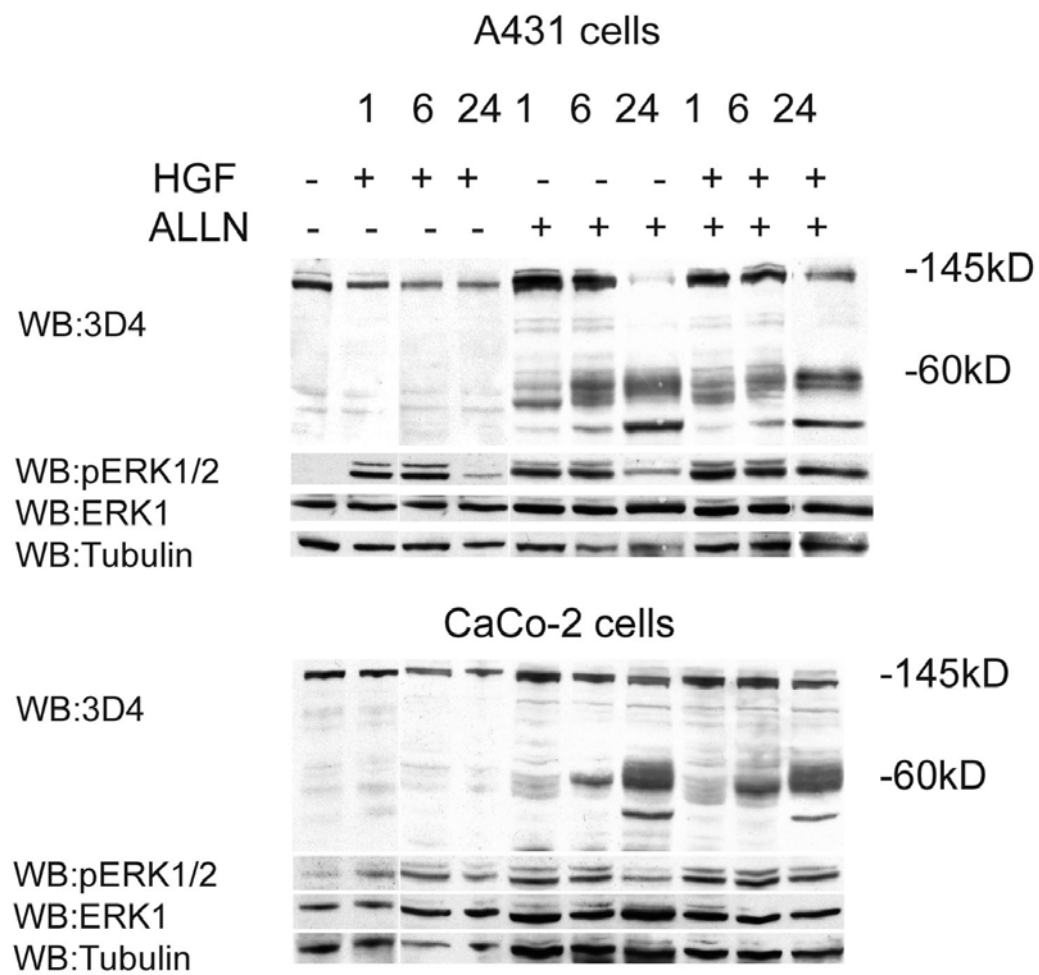


Figure 6a

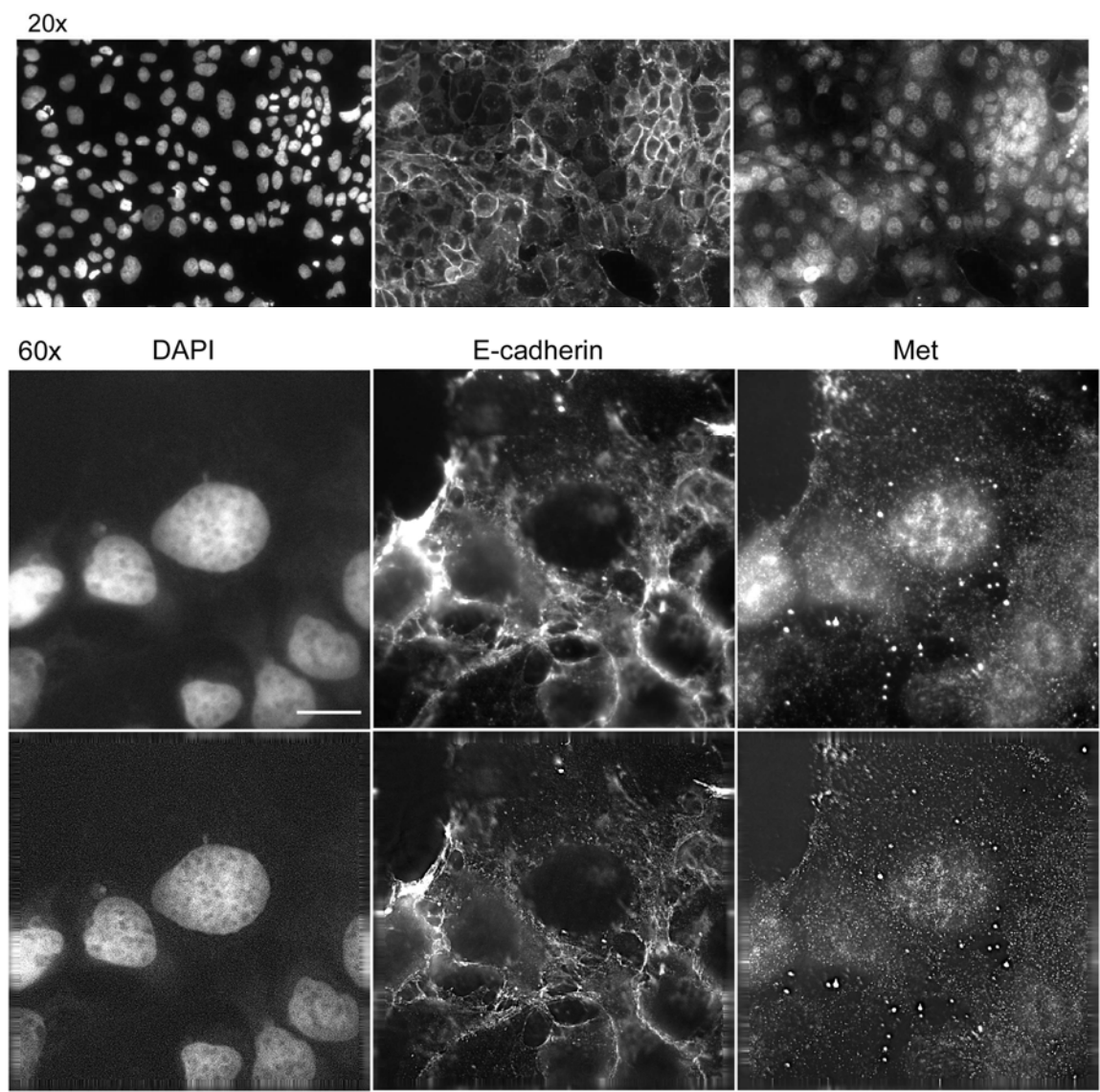
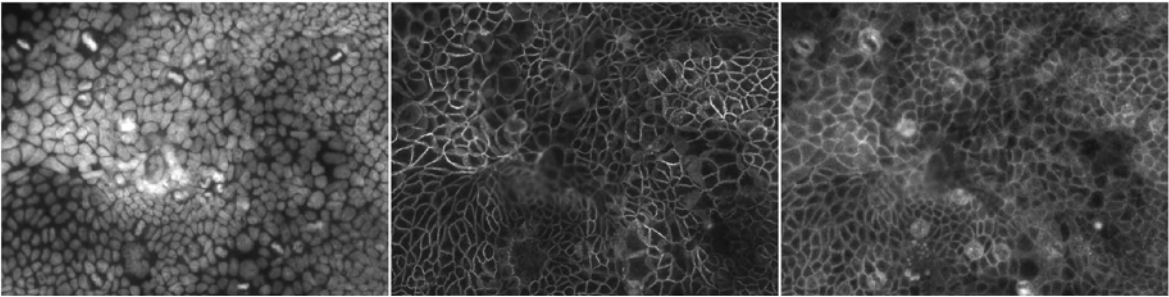


Figure 6b

20x



60x

DAPI

E-cadherin

Met

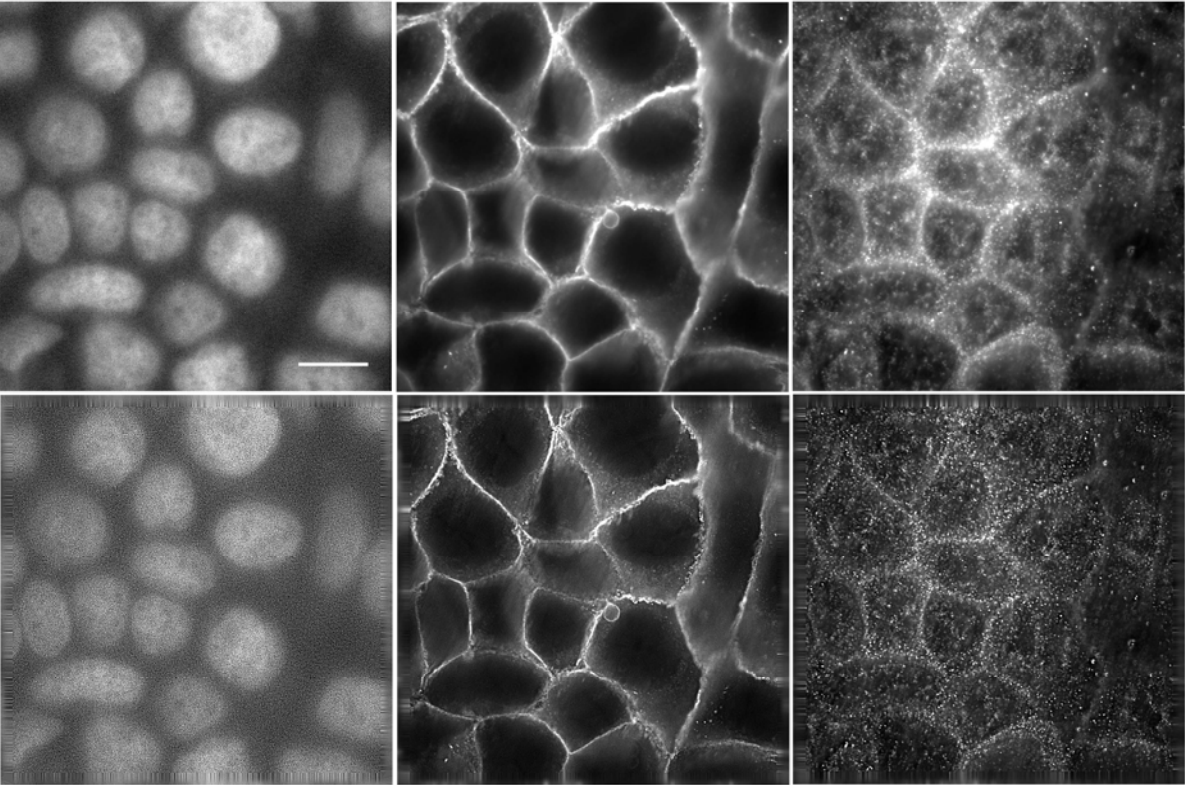


Figure 7

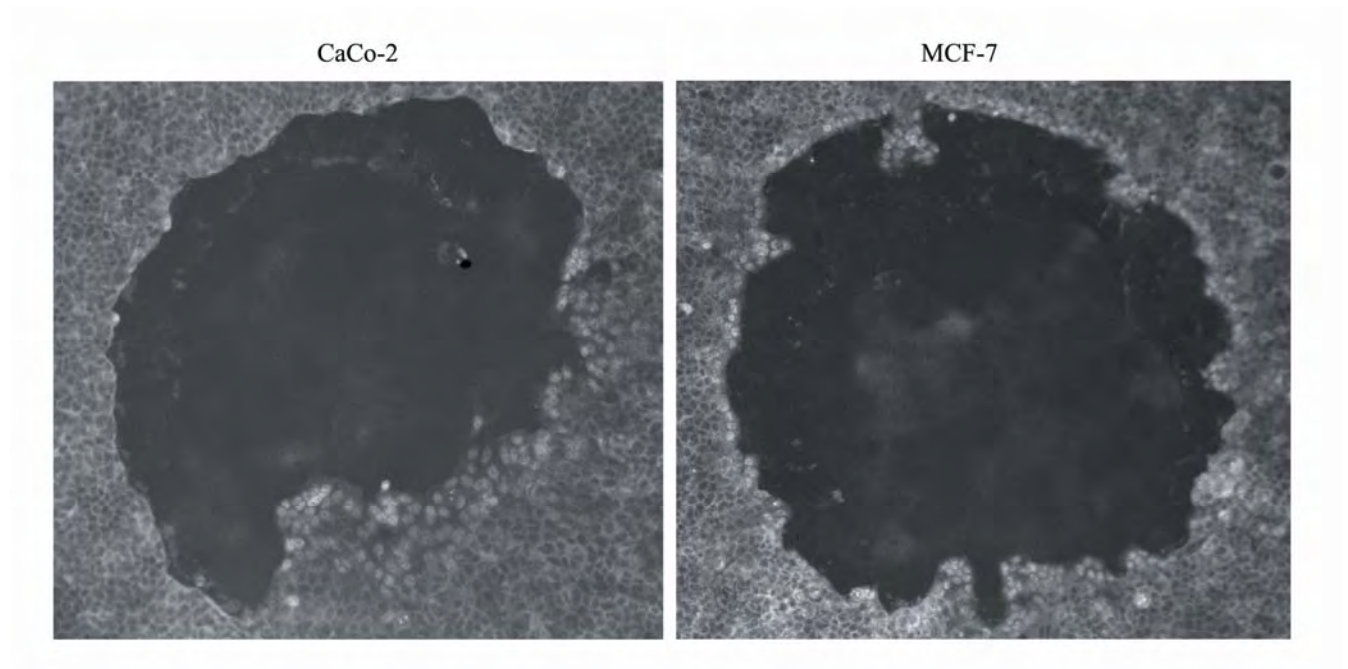


Figure 8

